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Identification of a functional serum response element in the HTLV-I LTR

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Abstract

In response to various mitogenic signals, serum response factor (SRF) activates cellular gene expression after binding to its cognate target sequence (CArG box) located within a serum response element (SRE). SRF is particularly important in T cell activation, and we now report that SRF activates basal transcription from the human T-cell leukemia virus-I (HTLV-I) long terminal repeat (LTR). A DNA element, with similarity to the consensus cellular CArG box found in the c-*fos* promoter centered approximately 120 base pairs upstream from the viral transcription start site, has been identified and named the vCArG box. SRF activation of gene expression from the LTR was localized to the vCArG box, and mutation of this site abolished SRF responsiveness. An oligonucleotide probe containing the vCArG box bound purified SRF, and a complex formed on this probe with nuclear extract was supershifted by anti-SRF antibody. Moreover, a biotinylated probe containing the vCArG box bound SRF in avidin–biotin pull-down assays. Quantitative binding analysis yielded nanomolar affinities for both the viral and cellular CArG boxes. Chromatin immunoprecipitation experiments demonstrated that SRF is resident on the HTLV-I LTR in vivo. These data identify a functional serum response element in the HTLV-I LTR and suggest that SRF may play an important role in regulating basal HTLV-I gene expression in early infection and reactivation from latency.

Keywords: HTLV-I; LTR; SRF; SRE; Basal transcription; T cell activation; Latency

Introduction

Serum response factor (SRF) is an immediate early (IE) 67 kDa cellular transcription factor that stimulates cell cycle entry in response to a variety of mitogenic signals. Originally identified as the protein responsible for enhancing c-*fos* gene expression (Norman and Treisman, 1988; Norman et al., 1988), SRF also activates expression of several immediate early genes such as *fosB*, *junB*, and *c-egr* (Herdegen and Leah, 1998; Treisman, 1995). SRF binds to a quasi-palindromic DNA sequence $CC(A/T)_6GG$, termed the CArG box, the prototype of which resides in the serum response element (SRE) of the c-*fos* promoter (Treisman, 1987). A complete SRE typically consists of a CArG box for SRF binding and an adjacent site that binds a member of the ternary complex transcription factor (TCF) family (Elk-1, Sap-1, or NET). (Dalton and Treisman, 1992; Janknecht and Nordheim, 1992). SRF is relatively abundant in T cells, and its activity is regulated in response to T cell activators at the posttranslational level through multiple strategies (Alberts and Treisman, 1998; Poser et al., 2000). The best characterized of these includes the Rho-dependent GTPase pathway (Hill et al., 1995).

Human T cell leukemia virus I (HTLV-I) was the first human pathogenic retrovirus identified (Poiesz et al., 1980) and is the etiologic agent of adult T-cell leukemia and a neurodegenerative disease, tropical spastic paraparesis/HTLV-I associated myelopathy (Gessain et al., 1985; Hinuma et al., 1981; Osame et al., 1986; Yoshida et al., 1982). Viral gene expression is regulated primarily through the promoter located in the 5′ viral long terminal repeat (LTR), and initial rounds of basal transcription and translation result in synthesis of Tax, the HTLV-I oncoprotein. Tax is a potent transactivator of the major viral promoter located in the 5′ LTR as well as the promoters of several cellular genes (De La Fuente et al., 2000; Mori et al., 2002; Nicot et al., 2000; Robert-Guroff et al., 1982). Tax is known to bind SRF and enhance its activation of certain

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cellular promoters (Alexandre and Verrier, 1991; Alexandre et al., 1991; Fujii et al., 1994, 1995a; Shuh and Derse, 2000; Suzuki et al., 1993). Following HTLV-I infection, immune responses to viral proteins are observed, but viral gene expression is very difficult to detect, suggesting that the virus is predominantly latent with intermittent bursts of gene expression (Robert-Guroff et al., 1982). Neither mechanisms that establish latency nor those that reactivate viral gene expression are well understood, although cellular transcription factors are likely involved (Mori et al., 1997; Newbound et al., 2000).

The viral LTR possesses several binding sites for cellular transcription factors. The three 21 bp imperfect repeats (TRE-1) bind CREB/ATF family members (Beimling and Moelling, 1992; Giam and Xu, 1989; Jeang et al., 1988; Yoshimura et al., 1990; Zhao and Giam, 1992). The central TRE-1 also binds Ap-1, and the proximal TRE-1 binds Sp1/Sp3 (Barnhart et al., 1997; Fujii et al., 1995b; Jeang et al., 1991; Wessner et al., 1997). AP-2 has also been shown to interact with each of the TRE-1 elements (Muchardt et al., 1992). A region denoted as TRE-2 resides between the central and proximal TRE-1s (Marriott et al., 1989, 1990)

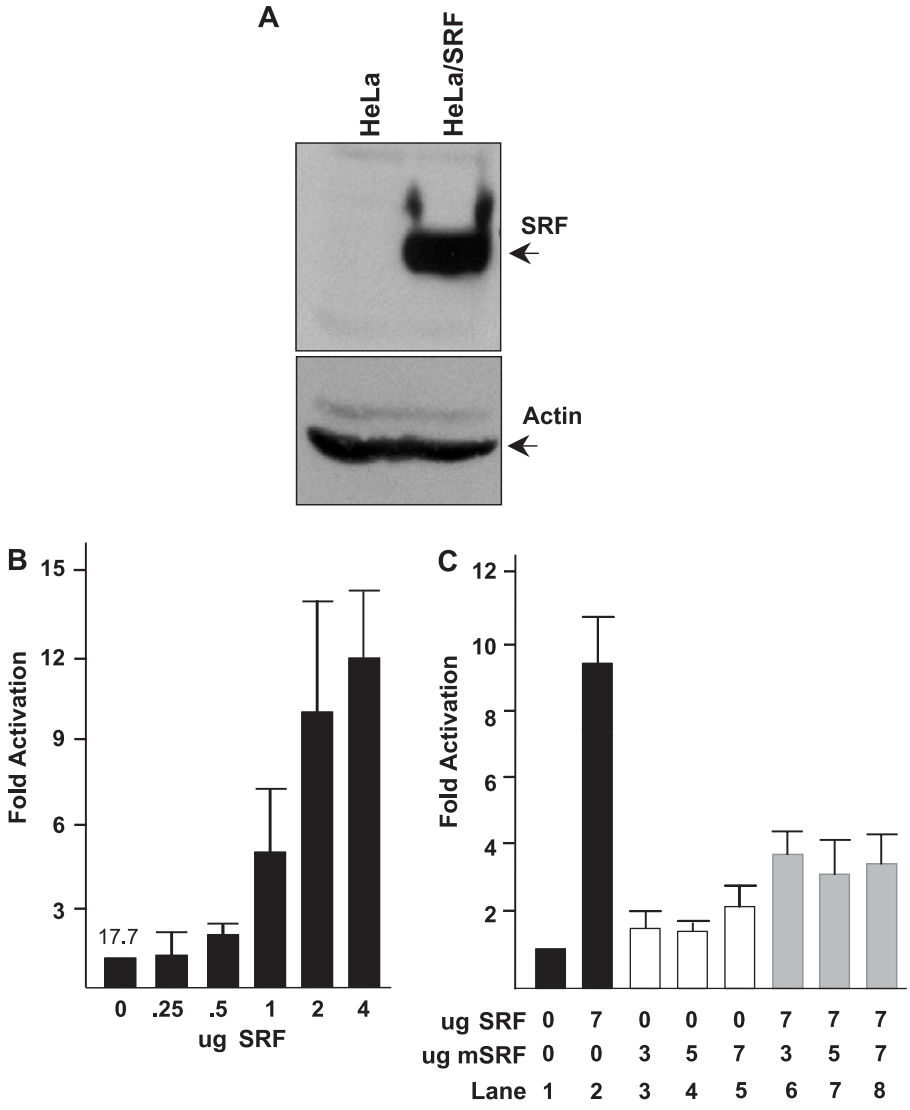


Fig. 1. SRF activates the HTLV-I LTR. (A) Analysis of SRF expression in HeLa cells. Whole-cell extracts were prepared from untransfected HeLa cells (left lane) and HeLa cells 48 h after transfection with pSG-SRF (right lane). SDS-PAGE followed by Western blot analysis employed 50 μg total protein in each lane. Top panel: probed with α-SRF. Bottom panel: probed with β-actin as a loading control. (B) Activation of the HTLV-I LTR by SRF. HeLa cells were transfected with 1 μg of pU3RLuc reporter alone, or with increasing concentrations of a plasmid encoding wild-type SRF (pCGN-SRF) as shown. The resulting luciferase activity was corrected for transfection efficiency based on the activity of cotransfected pRL-SV40 plasmid. Fold activation was calculated by dividing each corrected value by the corrected activity of pU3RLuc in the absence of cotransfected SRF. An average of three independent experiments is shown. The average corrected absolute activity is shown above the basal control bar. (C) Effects of a dominant negative SRF mutant on LTR activation. HeLa cells were transfected with 2 μg of pU3RLuc and combinations of wild-type (pCGN-SRF) and mutant (pCGN-SRFΔ5) SRF expression plasmids as shown. The results were corrected for transfection efficiency and fold activation was calculated as in panel B. The data presented are the average of two independent experiments.

and contains binding sites for multiple transcription factors including several from the Ets family and c-Myb (Bosselut et al., 1990, 1992; Clark et al., 1993; Soudant et al., 1994). Despite discovery of a wide variety of transcription factor binding sites in the LTR, the role of cellular proteins in regulating expression from the LTR remains incompletely characterized. Here we report identification and characterization of a functional serum response element within the HTLV-I LTR and propose that it may play a role in basal expression of the LTR as well as in activation of viral gene expression from latency.

Results

SRF activates basal transcription of the HTLV-I LTR

SRF is known to play an important role in regulating gene expression in T cells, often in concert with a member of the TCF transcription factor family. A TCF binding site has previously been identified in the HTLV-I LTR and was shown to regulate expression from the LTR (Bosselut et al., 1990; Gitlin et al., 1991). Because several viruses have been reported to contain functional SREs with binding sites for both SRF and a TCF family member, and because a TCF binding site has previously been identified within the HTLV-I LTR, we hypothesized that a functional SRE might also be present in the HTLV-I LTR. As an initial test of this hypothesis, the ability of SRF to activate transcription from the HTLV-I LTR was investigated by transient transfection of an LTR reporter plasmid into HeLa cells, which contain minimal levels of endogenous SRF (Fig. 1A). Increasing amounts of transfected SRF activated LTR-dependent reporter expression in a dose-dependent manner to a maximum of 12-fold (Fig. 1B).

To confirm the specificity of this response, a dominant-negative SRF mutant was tested for its ability to inhibit SRF activation of the LTR. Although a deletion in its activation domain renders it unable to activate transcription, this mutant retains the dimerization and DNA-binding capabilities of wild-type SRF, allowing it to inhibit the transcriptional activity of wt SRF (Belaguli et al., 1999). In these experiments, wild-type SRF activated the LTR approximately ninefold (Fig. 1C, lane 2), whereas increasing amounts of the dominant negative mutant alone failed to alter transcription from the LTR significantly (lanes 3–5). Because transfection of the dominant negative SRF alone did not reduce reporter expression below that seen in the absence of SRF (compare lane 1 with lanes 3–5), these results indicate that the level of endogenous SRF in HeLa cells is not sufficient to activate the LTR. Cotransfection of the dominant negative SRF mutant with wild-type SRF decreased SRF-activated transcription of the LTR approximately threefold (lanes 6–8), confirming the dominant negative phenotype of the mutant SRF. Together, these data demonstrate that SRF is specifically capable of stimulating transcription from the HTLV-I LTR.

Localization of an SRF response element within the HTLV-I LTR

To identify the region within the LTR that confers SRF responsiveness, transfection experiments were performed using a panel of LTR deletion mutants. This series of reporter plasmid constructs consists of incremental deletions of 5′ LTR sequence driving CAT expression (Brady et al., 1987) (Fig. 2). Basal expression of the full-length promoter was four- to fivefold higher (11.4 compared with 1.8, 3.5, and 2.0) than the deletion mutants that contained at least one TRE 1, implying the presence of transcriptional elements upstream of the distal TRE1. SRF

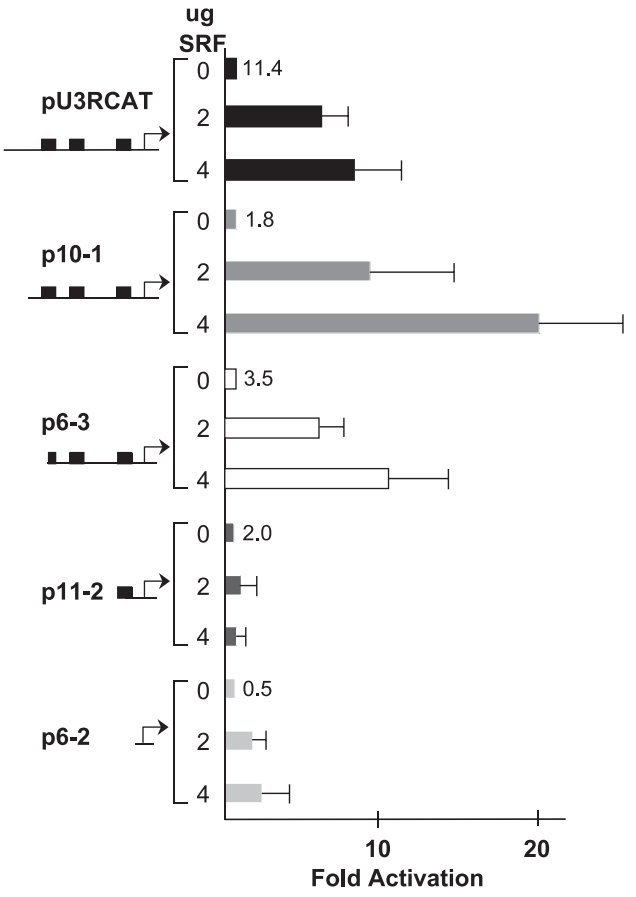


Fig. 2. Analysis of LTR deletion mutants for SRF activation. The wild-type LTR (pU3RCAT) and a series of 5′ LTR deletion mutants (p10-1, p6-3, p11-2, and p6-2) are schematically depicted to the left. The black rectangles represent the three TRE-1s, shown for orientation purposes. These constructs were transfected into HeLa cells alone or with 2 or 4 μg of pCGN-SRF. The resulting CAT activities were corrected for transfection efficiency based on the activity of cotransfected pSV-β-gal plasmid. Fold activation was calculated by dividing the corrected activity in the presence of SRF by that in the absence of cotransfected SRF for each vector. The data presented are the average of four independent experiments. The average corrected absolute reporter activity is displayed to the right of each basal reporter bar.

activated the full-length LTR, mutant p10-1, and mutant p6-3 from 10- to 20-fold. In contrast, mutants p11-2 and p6-2 were not significantly activated by SRF. These data localize a potential SRF response element to a sequence between the 5′ ends of mutants p6-3 and p11-2, corresponding to bases −244 to −101 relative to the transcription start site.

The HTLV-I LTR contains a specific SRF binding site

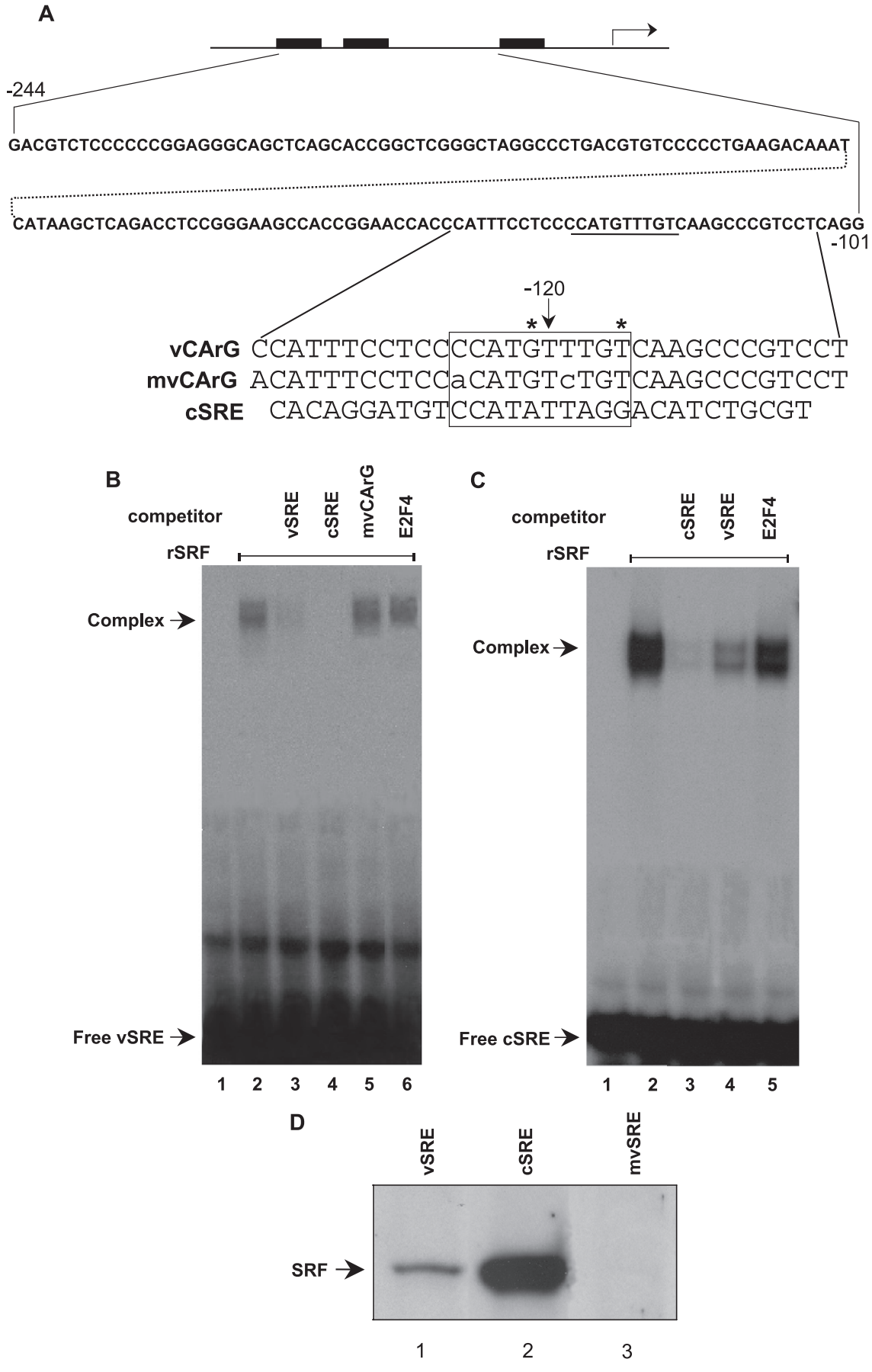
To identify an SRF responsive element between bases −244 to −101, this sequence was submitted to Transcriptional Element Search Software (TESS on the web, http://www.cbil.upenn.edu/tess/) for analysis (Schug and Overton, 1997). This algorithm identified a 10-base sequence centered at −120 that we refer to as the viral CArG box (vCArG) with 80% similarity to the c-*fos* CArG box (Fig. 3A, underlined). The sequence of the HTLV-I vCArG box [CCATGTTTGT] deviates from the canonical c-*fos* CArG box [CC(A/T)$_6$GG] (Leung and Miyamoto, 1989; Norman and Treisman, 1988; Rivera et al., 1990) by the presence of a single G residue within the A/T-rich core and a T residue in the 3′ most position (noted by asterisks). The vCArG also contains a T in place of an A in the eighth position of the c-*fos* CArG (cSRE, shown for comparison), but this substitution retains the A/T-rich nature of the CArG box. In the following experiments, oligonucleotides corresponding to viral sequences that contain the vCArG box as well as a predicted TCF binding site are referred to as the viral serum response element (vSRE).

To examine SRF-binding ability, the vSRE was used as an oligonucleotide probe in EMSA experiments. Incubation with purified recombinant SRF resulted in formation of a shifted complex (Fig. 3B, lane 2). The specificity of this complex was confirmed by competition with unlabeled self (lane 3) as well as by an unlabeled oligonucleotide containing the canonical CArG box located within the c-*fos* promoter SRE (cSRE, lane 4). The complex was not competed when a mutation was introduced into the vSRE CArG box (lane 5, mvCArG, sequence shown in Fig. 3A). A binding site for the unrelated E2F4 transcription factor (lane 6) was also unable to compete for formation of the complex. The E2F4 binding site competitor was capable of binding E2F4 when labeled and used as a gel shift probe with HeLa nuclear extract (data not shown). To extend these findings and compare the vSRE to the cSRE, similar experiments were performed using cSRE as a probe. A shifted complex, comparable to that formed on the vSRE, was seen in the presence of SRF (Fig. 3C, lane 2). Both unlabeled cSRE (lane 3) and, to a lesser extent, vSRE (lane 4) competed for SRF binding to the cSRE probe. The relative levels of competition suggested that the vSRE might be a lower-affinity binding site than the cSRE (compare lanes 3 and 4). As expected, the E2F4 binding site competitor (lane 5) did not compete for binding.

The assembly of SRF on the vSRE in vitro was confirmed using biotin-labeled vSRE and cSRE oligonucleotides in an avidin–biotin pull-down assay. The biotinylated vSRE and cSRE probes bound recombinant SRF (Fig. 3D, lanes 1 and 2, respectively). The mvCArG binding site oligonucleotide, which did not compete for SRF binding in the EMSA assays, did not precipitate SRF in this assay (lane 3). Although equal molar amounts of the vSRE and cSRE oligonucleotides were used in the avidin–biotin pull-down assay, more SRF was recovered on the cSRE oligonucleotide than on the vSRE, consistent with the EMSA results in Fig. 3C and implying reduced affinity of the vSRE for SRF compared to the cSRE.

To evaluate the ability of the vSRE probe to bind SRF within a complex protein mixture, EMSA experiments were performed using nuclear extract (HeLa/SRF) from HeLa cells that had been transiently transfected with an SRF expression plasmid. SRF expression in the HeLa/SRF extracts was confirmed by Western blot (Fig. 1A). HeLa/SRF nuclear extract formed a shifted complex on the vSRE probe (Fig. 4A, lane 2). Specificity of the complex was verified by competition with self (lane 3) as well as by competition with the cSRE (lane 4). An oligonucleotide containing the E2F4 binding site was unable to compete for binding (lane 5). An EMSA supershift verified that the complex contained SRF (Fig. 4B). As previously, addition of nuclear extract resulted in a shifted complex (lane 2). Addition of anti-SRF antibody supershifted the complex (lane 3), although an unrelated isotype-matched antibody to Sp1 did not (lane 4). The results shown in panels A and B demonstrate that the vSRE forms a specific complex with protein(s) from nuclear extracts that can be supershifted by anti-SRF antibody. HeLa extracts that had not been transfected with an SRF expression plasmid failed to show binding (data not shown), supporting the results in Fig. 1 showing that HeLa cells contain insufficient concentrations of SRF to bind and activate the HTLV-I LTR.

Because both the avidin–biotin pull-down experiments and the competition reactions from EMSA experiments suggested a difference in cSRE and vSRE affinities for SRF, we examined those affinities by quantitative nitrocellulose filter binding experiments. Oligonucleotides containing the vCArG (vSRE-39) or the cSRE were incubated with varying concentrations of purified recombinant SRF (Fig. 5). Fitted curves for these experiments using Eq. (1) (Materials and methods) determined a K_d of 1.1×10^{-9} M for cSRE binding to SRF (Panel A) and a K_d of 6.7×10^{-9} M for vSRE-39 binding to SRF (panel B) with little or no cooperativity. The difference in binding affinity of approximately sixfold likely reflects the variation in vSRE-39 sequence from the canonical cSRE sequence and confirms the EMSA binding and avidin-biotin pull-down data. Because these experiments employed recombinant SRF, it should be noted that posttranslational modifications of SRF occurring in vivo may affect its binding affinities at these sites.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CATAAGCTCAGACCTCCGGGAAGCCACCGGAACCACCCATTTCCTCCCCATGTTTGTCAAGCCCGTCCTCAGG
-101
-120
vCArG CCATTTCCTCCCCATGTTTGTCAAGCCCGTCCT
mvCArG ACATTTCCTCCaCATGTcTGTCAAGCCCGTCCT
cSRE CACAGGATGTCCATATTAGGACATCTGCGT
B
competitor
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mvCArG
E2F4
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Complex
Free vSRE
1 2 3 4 5 6
C
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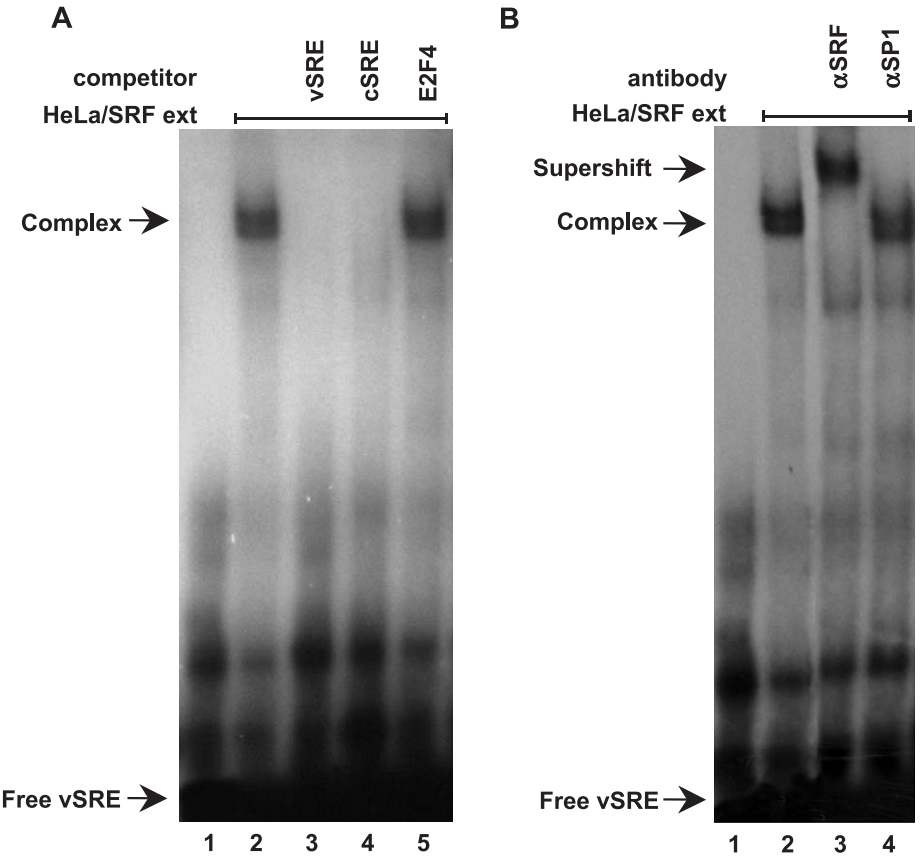

Fig. 4. The vCArG box binds SRF in nuclear extracts. (A) EMSA analysis of HeLa/SRF extracts (lanes 2–5) and ^{32}P-labeled vSRE probe (lanes 1–5). Lane 3, competition with vSRE; lane 4, competition with cSRE; lane 5, competition with E2F4. (B) EMSA supershift analysis with HeLa/SRF extract (lanes 2–4) and vSRE probe (lanes 1–4). Lane 3, supershift with anti-SRF antibody; lane 4, supershift with anti-Sp1 antibody.

The HTLV-I vCArG is necessary for SRF transactivation of the LTR

To examine whether SRF binding to the vSRE modulates activation of the LTR, a two base pair mutation in the vCArG (Fig. 6A) was introduced within the context of the complete LTR by site-directed mutagenesis (mvCArG LTR). This mutation corresponded to that used in EMSA experiments with recombinant SRF (shown in Fig. 3B). Transfection of HeLa cells with the wild-type LTR and increasing concentrations of SRF resulted in increased LTR activity (Fig. 6B) as seen previously. Conversely, cotransfection of mvCArG LTR with increasing amounts of SRF showed no increased activity above background, indicating that the mutation abolished the capacity of the LTR to respond to SRF. As seen in HeLa cells, Jurkat T cells express relatively low levels of SRF, and analogous transfection experiments in the Jurkat human T cell line resulted in a similar pattern of activation (Fig. 6C), with increasing amounts of SRF activating the wild-type LTR reporter, but not the mvCArG LTR reporter. These results demonstrate that the vCArG sequence is specifically required for the wild-type LTR to respond to SRF.

To verify that the inability of mvCArG to respond to SRF was specific to the mutation rather than to a gross defect in the plasmid, its responsiveness to CREB was

Fig. 3. The HTLV-I LTR contains a vCArG that binds SRF. (A) LTR sequence encompassing the SRF responsive region (−244 to −101) within the HTLV-I LTR. The position of the SRF responsive region within the LTR is indicated relative to the transcription start site and the three TRE-1s (black boxes). TESS sequence analysis of the responsive region identified an element with homology to a consensus CArG box-binding site centered at residue −120 relative to the transcription start site, the vCArG (underlined). For comparison, both the mutant vCArG (mvCArG), containing two mutations (denoted as lower case letters) relative to the wild-type vCArG and the cSRE sequences are shown below, with the 10 base CArG element boxed. Asterisks note the nonhomologous bases of the vCArG relative to the cCArG. (B) EMSA of vSRE probe with purified, recombinant SRF. Recombinant SRF at 2×10^{-7} M (lanes 2–6) was incubated with $2–5 \times 10^{-10}$ M probe (lanes 1–6). Competitor sequences were added in 250-fold excess as indicated (lane 3, vSRE; lane 4, cSRE; lane 5, mvCArG; lane 6, E2F4). (C) EMSA of cSRE probe with purified, recombinant SRF. Recombinant SRF at 2×10^{-7} M (lanes 2–5) was incubated with 2×10^{-10} M probe (lanes 1–5). Competition analysis was performed as described in panel B (lane 3, cSRE; lane 4, vSRE; lane 5, E2F4). (D) vSRE binds recombinant SRF in avidin–biotin pull-down assay. Recombinant SRF was incubated with biotin-labeled vSRE (lane 1), cSRE (lane 2), or mvSRE (lane 3) double-stranded oligonucleotides. Complexes were collected on immobilized streptavidin agarose beads and analyzed by Western blot with anti-SRF antibody. A constant amount of protein and equal molar amounts of biotinylated oligonucleotides were used in each sample.

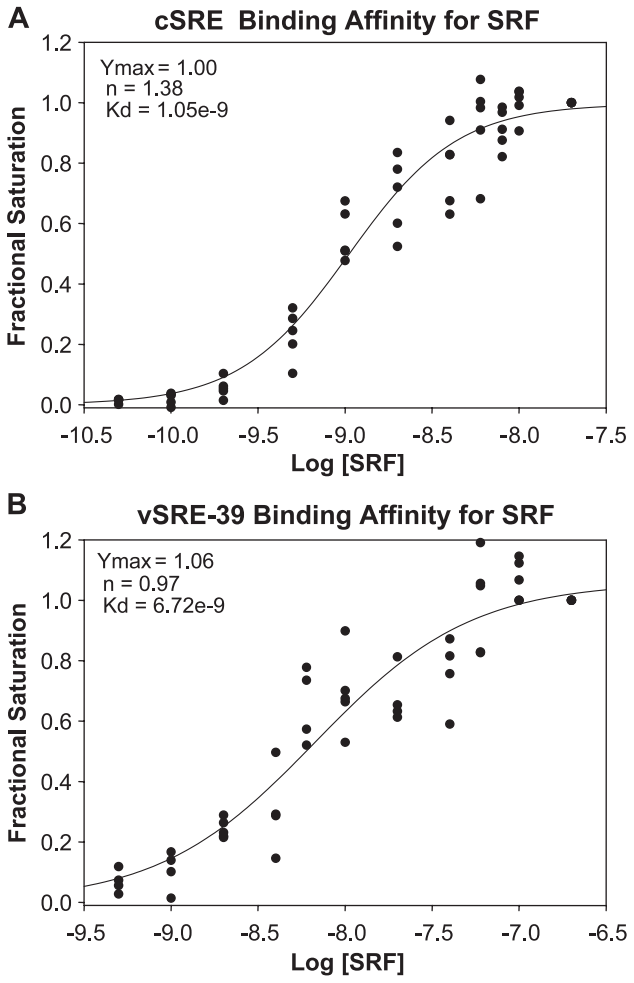

Fig. 5. Affinity of SRF binding to the cCArG and vCArG boxes. Increasing amounts of purified recombinant SRF were incubated with ^{32}P-labeled cSRE or vSRE-39, and reactions were analyzed by nitrocellulose filter binding. Signal was detected by phosphor-imager analysis, and data were quantitated with ImageQuant software. Data were fit to Eq. (1) by nonlinear least squares analysis in SigmaPlot 2000. (A) Graph of cSRE binding data with fitted curve shown. These data are from four independent experiments with replicates. (B) Graph of vSRE binding data with fitted curve shown. These data are from five independent experiments with replicates. The data for SRF binding to cSRE fit with a K_d of 1.1×10^{-9} M, and the data for SRF binding to vCArG fit with a K_d of 6.7×10^{-9} M.

examined in transfection experiments similar to those above. The LTR is activated by CREB in the presence or absence of PKA phosphorylation (Beimling and Moelling, 1992; Kwok et al., 1996). Wild-type or mvCArG LTR reporter plasmids were transfected into HeLa cells in the presence or absence of a CREB expression plasmid and in the presence or absence of a PKA expression plasmid (Fig. 6D). Both wild-type and mvCArG LTRs were weakly activated by CREB or PKA independently, and were equally activated three- to fourfold by CREB and PKA together, indicating that the lack of responsiveness of the mvCArG LTR was specific to SRF and that mvCArG retains competence for transcriptional activation by other transcription factors.

SRF is associated with the HTLV-I LTR in vivo

To extend the in vitro binding results from the EMSA and avidin–biotin pull-down experiments described above and to correlate LTR activation seen in transfection experiments with SRF binding, we examined the binding of SRF to the LTR in vivo by chromatin immunoprecipitation. In these experiments anti-SRF antibody was used to immunoprecipitate protein that had been chemically cross-linked with chromatin in three T cell lines, HuT 102, which contains several integrated copies of the HTLV-I LTR; MS9, which contain a single integrated copy of the HTLV-I LTR, and Jurkat, which do not contain the HTLV-LTR. The coprecipitated, purified DNA was then used as a template in PCR reactions with HTLV-I LTR-specific primers that flank the vSRE (Fig. 7A, panel 1). Anti-SRF antibody precipitated the LTR cross-linked to SRF protein from HuT102 cells, whereas both a nonspecific antibody (rabbit IgG) and a control reaction in the absence of antibody did not precipitate LTR DNA. Similar results were obtained using the infected cell line, MS9 (Hill et al., 1999; Shuh et al., 1999), containing a single integrated HTLV-I provirus (Fig. 7A, panel 2). No nonspecific DNA band was detected by PCR amplification of template DNA purified from uninfected Jurkat cells subjected to the same chromatin immunoprecipitation (Fig. 7A, panel 3).

To verify the functionality of ChIP studies in Jurkat cells, anti-SRF immunoprecipitated DNA was analyzed by amplification with c-*fos* promoter specific primers (Fig. 7B). Whereas DNA purified from the anti-SRF immunoprecipitation was amplified using c-*fos* primers, neither DNA from a no-antibody control nor that from nonspecific antibody (rabbit IgG) immunoprecipitation was. Collectively, these data demonstrate that SRF can bind specifically to the HTLV-I LTR in vivo.

Discussion

These experiments have identified an SRF-responsive element within the HTLV-I LTR. This SRE contained a CArG box that was localized using a series of LTR deletion mutant reporter constructs in transfections and was tentatively identified by sequence homology to the canonical c-*fos* CArG box. The capacity of the vCArG box to bind SRF in vitro was confirmed by EMSA experiments using recombinant SRF and nuclear extracts as well as with avidin–biotin pull-down assays. SRF was also shown to bind the LTR in vivo by chromatin immunoprecipitation. Functionally, the ability of SRF to activate the viral SRE in vivo was demonstrated by transient transfection experiments that employed two approaches. First, a role for SRF protein in LTR regulation was demonstrated using a dominant negative mutant SRF that inhibited SRF activation of the LTR. Then the vCArG sequence was shown to be required for

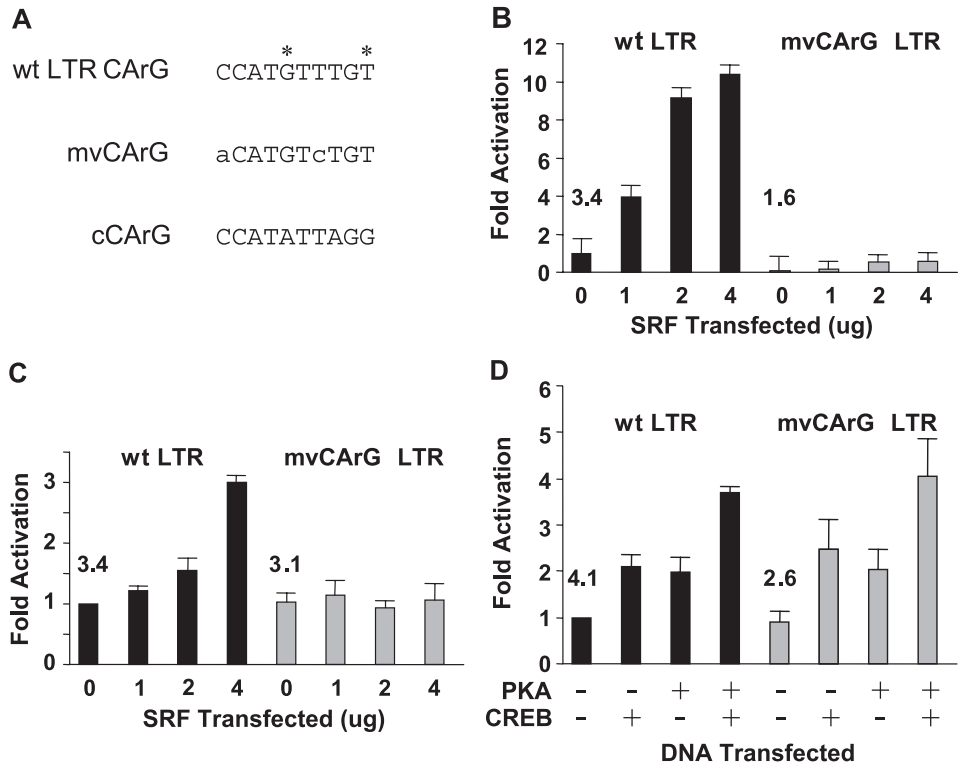

Fig. 6. Mutation of the vCArG abrogates SRF activation of the HTLV-I LTR. (A) Viral LTR CArG and mutant viral LTR CArG sequences are shown here. Positions at which the viral CArG sequence varies from the canonical cellular sequence are noted by asterisks. Mutations introduced into the wt viral CArG to make mvCArG are noted by lower case letters. The cellular c-*fos* CArG box is shown for comparison. (B) HeLa cells were transfected with reporter plasmids containing either wild-type LTR (black bars, wt CArG sequence shown in panel A) or LTR containing mutations within the vCArG (mvCArG, gray bars, sequence shown in panel A) along with increasing amounts of pSG-SRF expression plasmid as indicated. The numbers above the bars show the average corrected absolute reporter activity in the absence of SRF. Fold activation was determined as in Fig. 1. These data are the average results of three independent experiments. (C) Jurkat lymphocytes were used in transfection experiments similar to those in panel B. The numbers above the bars show the average corrected absolute reporter activity in the absence of SRF. These data are the average results of three independent experiments. (D) HeLa cells were transfected with reporter plasmids containing either wild-type LTR (black bars) or mvCArG (gray bars) along with PKA and/or CREB expression plasmids as indicated. Activity was corrected for transfection efficiency, and fold activation was calculated as described in Materials and methods. The numbers above the bars show the average corrected absolute reporter activity in the absence of SRF. These data are the average results of three independent experiments.

SRF activation of the LTR using an LTR reporter plasmid containing a mutation in the vCArG box.

The viral CArG box displayed a reduced apparent binding affinity of about sixfold for SRF relative to that of the cSRE in the in vitro binding studies. This difference probably reflects variance between the vCArG and cCArG sequences. The presence of a C/G pair within the A/T-rich region of the CArG box, similar to the vCArG sequence, has previously been reported to reduce the affinity of SRF binding up to tenfold (Pollock and Treisman, 1990). Our quantitative analysis of SRF protein binding affinity yielded a K_d of 1.1×10^{-9} M for the cSRE and a K_d of 6.7×10^{-9} M for the vCArG, values well within the range for specific DNA–protein interaction affinities. In vivo, binding affinities are probably modulated differentially at the two promoters by posttranslational modifications and by interactions with different cofactors and accessory proteins. The binding of wild-type SRF to the LTR is consistent with transcriptional activation of the LTR by SRF in vivo.

The presence of a functional SRE within the HTLV-I promoter may provide an important strategy for the virus. SRF protein in T cells is activated upon T cell stimulation (Magnaghi-Jaulin et al., 1996). The binding of HTLV-I particles to the cell surface activates T cells through the CD2 pathway, initiating a series of events that may ultimately propel the cell into the cell cycle (Dodon et al., 1989; Sotiropoulos et al., 1999). In response to T cell activation, SRF becomes transcriptionally activated either as a downstream target of signaling pathways such as Rho GTPase (Hill et al., 1995) or by binding to specific protein partners (Chen et al., 1996; Davis et al., 2003; Hanlon et al., 2001; Natesan and Gilman, 1995; Sotiropoulos et al., 1999). Following activation, SRF induces transcription of several genes, including c-*fos* and *egr-1* (Poser et al., 2000; Schratt et al., 2001), that promote cell cycling. These events could promote translocation of the viral genome into the nucleus and subsequent chromosomal integration as a provirus (Gineitis and Treisman, 2001; Poser et al., 2000). Our data are consistent with the possibility that increased SRF activity in an infected cell as a result of T cell activation may stimulate transcription of the newly integrated proviral genome via the vSRE. Thus, HTLV-I may use SRF to meet

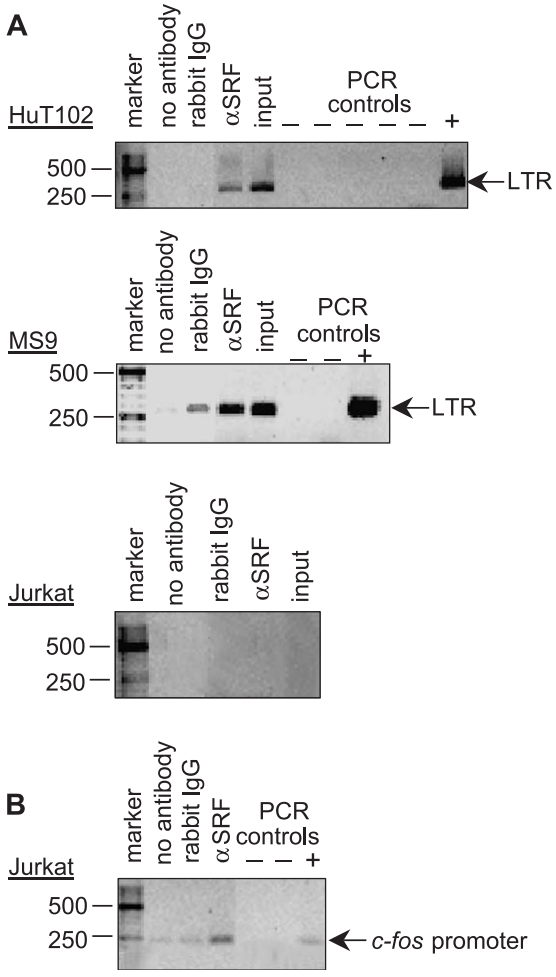

Fig. 7. SRF binds the HTLV-I LTR in vivo. (A) Cross-linked Jurkat (LTR absent), HuT102 (LTR present), and MS9 (single LTR present) nuclear lysates were subjected to immunoprecipitation with protein-G beads bound with anti-SRF antibody. Cross-links were reversed, and purified DNA was used as template in PCR reactions with LTR-specific primers followed by agarose gel electrophoresis analysis of PCR products. Negative controls included ChIPs with no antibody and with rabbit IgG. Input DNA purified from 1/10 of the amount of HuT102 or MS9 lysate used for IPs was amplified by PCR as a positive ChIP control. Negative control PCR samples included no template or primers, no 3′ primer, no 5′ primer, no primers, and no template. Amplification of an HTLV-I proviral clone DNA template served as the positive PCR control. (B) PCR analysis of Jurkat ChIPs with c-*fos* promoter-specific primers. DNA samples precipitated with the same anti-SRF antibody as in panel A were amplified with the c-*fos* promoter specific primers. PCR controls included no template, no primers, and as positive PCR control, the plasmid template pc-fos-CAT.

two requisites of the early infecting virus: activation of cell division and stimulation of viral transcription. Similarly, T cell activation resulting in SRF activation may promote the release from viral latency at later stages of infection. Subsequent translation of viral mRNA yields Tax protein. Tax has been shown to interact with SRF at cellular promoters (Fujii et al., 1988, 1992, 1994), although the ability of SRF to recruit Tax to the HTLV-I LTR has not yet been analyzed. Future studies will address this very important issue.

Serum response elements typically contain a CArG box adjacent to a binding site for a member of the ternary complex factor (TCF) subfamily of the Ets transcription factor family. SRF can activate the SRE in vivo by binding independently to the CArG box (Price et al., 1996; Williams and Lau, 1993). However, the binding of SRF in conjunction with a TCF can enhance the transcriptional response. At the c-*fos* promoter, as well as at the promoters of other SRF-responsive genes, three TCF proteins, Elk-1, Sap-1a, and NET, have been identified as part of a ternary complex with SRF (Clarkson et al., 1999; Price et al., 1996). Another Ets family member, Fli-1, has also been reported to bind the c-*fos* promoter and to interact with SRF (Dalgleish and Sharrocks, 2000). Non-Ets proteins such as steroid receptor coactivator-1 (SRC-1) (Kim et al., 1998), C/EBPβ (Hanlon et al., 2001), and GATA proteins (Morin et al., 2001) also bind to the c-*fos* SRE and cooperate with SRF in transcriptional activation. Although Ets proteins have been shown to bind the HTLV-I LTR in proximity to the vCArG (Bosselut et al., 1990; Gitlin et al., 1991), the role if any of TCFs, other Ets family members, or other co-accessory proteins in SRF activation of LTR expression remains to be established.

A cadre of coactivators, including CBP, p300, and P/CAF, has been implicated in transcription of a variety of promoters. CBP is recruited to the CArG box of the c-*fos* promoter and functions to stimulate promoter activity. Occupation of the Ets binding site is not required for CBP activation of the c-*fos* promoter (Ramirez et al., 1997). An alternative coactivator, ASC-2, also interacts with SRF at the c-*fos* promoter and can stimulate transcription alone or in combination with SRC-1 or p300 (Lee et al., 2000). We know that CBP/p300 play important roles in Tax transactivation of the LTR (Harrod et al., 2000; Kwok et al., 1996). The ability of SRF and other basal factors to recruit coactivators to the LTR may make them more readily available for use by the activated transcription complex.

Our identification of a serum response element within the HTLV-I LTR implies a previously unknown role for the cellular SRF protein in regulation of HTLV-I transcription. These results have expanded our understanding of viral strategies to accomplish basal transcription. Identification of accessory molecules and protein interactions important for function and regulation of the SRF complex will increase our understanding of HTLV-I gene expression, particularly basal activity and activation from latency. As a more complete picture of early events in viral infection emerges, new therapeutic strategies to subvert HTLV-I infection may be developed.

Materials and methods

Plasmids

The SRF (pCGN-SRF), mutant SRF (pCGN-SRFΔ5), and GST-conjugated SRF (pGST-SRF) expression plas-

mids were provided by Robert Schwartz (Baylor College of Medicine) and have been described previously (Belaguli et al., 1999; Chen and Schwartz, 1996). The expression plasmid pSG-SRF, provided by Masahiro Fujii (Niigata University School of Medicine), expresses SRF from an SV40 promoter (Fujii et al., 1992). The reporter plasmid pU3RLuc was constructed by ligating a 755 base pair *Xho* I–*Hin*d III LTR fragment from pU3RCAT (Sodroski et al., 1984) into the pGL3-Basic vector, purchased from Promega. Plasmids p6-2, p11-2, p10-1, and p6-3 have been described previously (Brady et al., 1987). Plasmids pSV-β-galactosidase and pRL-SV40 were purchased from Promega. Plasmid pCMVTax has been described previously (Rimsky et al., 1988). Plasmid pc-fos-CAT has been described previously (Lenardo et al., 1987). Plasmid pRSV-CREB and pWT-PKA were gifts of Marc Montminy (The Salk Institute for Biological Studies).

Oligonucleotides

Oligonucleotides were synthesized by Sigma-Genosys or Integrated DNA Technologies. The c-*fos* SRE probe used for both qualitative and quantitative binding analysis contained the sense sequence 5′-GATCAGATCCAGGATGTCCATATTAGGACATCTGT-3′. The vSRE-39 probe used for quantitative analysis contained the wt sense sequence 5′-GATCCGGACATTTCCTCCCCATGTTTGTCAAGCCGTCCT-3′. The vSRE probe used for qualitative EMSA experiments and avidin–biotin pull-down assays contained the wt sense sequence 5′-GATCCGGAGACCTCCGGGAAGCCACCAAGAACCACCCATTTCCTCCCCATGTTTGTCAAGCC-3′ and its mutant contained the sense sequence 5′-GATCCGGAGACCTCCGGGAAGCCACCAAGAACCACCCATTTCCTCCaCATGTcTGTCAAGCC-3′ (mutations shown in lower case). The following oligonucleotides were used as primers for amplification of the LTR in ChIP assays: upstream, 5′-GAAGTCTGAGAAGGTCAGGG-3′, and downstream, 5′-CCACGCTTTTATAGACTCCTG-3′. The following oligonucleotides were used as primers for amplification of the c-*fos* promoter: upstream, 5′-ACCCTCGGTGTTGGCTG-3′, and downstream, 5′-TCCTAATCTCGTGAGCATTTCG-3′. The following oligonucleotides were used as PCR mutagenesis primers: sense (mutation containing), 5′-GGAGGAAATGGGTGGTTCTTG-3′, and anti-sense, 5′-GGAGGAAATGGGTGGTTCTTG-3′.

Cell lines

HeLa cells were maintained in Dulbecco's modified eagle medium (DMEM) supplemented with 10% fetal calf serum. The Jurkat, HuT102, and MS9 cell lines were maintained in RPMI medium supplemented with 10% fetal calf serum. MS9 cells, provided by David Derse (National Cancer Institute, Frederick, MD), are HTLV-I infected and were grown in the presence of 100 U/ml IL2 in culture (Shuh et al., 1999).

Transfections and reporter assays

HeLa cells were transfected using Fugene 6 reagent (Roche) according to manufacturer's instructions or the calcium phosphate method as described previously (Connor et al., 1993). DMRIE-C reagent (Life Technologies) was used to transfect Jurkat cells according to manufacturer's instructions. Luciferase activity was assayed using the Luciferase Assay Kit or Dual Luciferase Assay Kit (Promega) and measured with a Turner TD-20e luminometer. CAT assays were performed as previously described (Connor et al., 1993). β-gal assays were performed using the modified 96-well plate method (Rosenthal, 1987). Indicated results were corrected for transfection efficiency by dividing luciferase units by β-gal units or by Renilla luciferase units from cotransfections with SV-β-gal or pRL-SV40, respectively. Fold activation was calculated by dividing each corrected activity by the corrected basal activity of the same reporter.

Expression and purification of SRF

The GST-SRF fusion protein was expressed in *E. coli* and purified on glutathione beads according to manufacturer's instructions (Amersham) with some modifications. Briefly, bacterial pellets resuspended in lysis buffer (100 mM Tris–HCl, pH 7.5; 200 mM KCl; 10 mM DTT; 10 mM $MgCl_2$) and 40 μg/ml lysozyme were frozen overnight, thawed slowly, and sonicated twice for 15 s. Soluble protein was recovered by centrifugation and subjected to 40% ammonium sulfate precipitation. The precipitate was resuspended in dialysis buffer (50 mM Tris-HCl pH 7.5; 100 mM KCl; and 10% glycerol) and dialyzed against the same buffer overnight. After centrifugation, the dialysate was loaded onto a column of glutathione sepharose 4B resin (Amersham Pharmacia). After washing, GST-SRF was eluted with glutathione elution buffer (25 mM reduced glutathione, 150 mM KCl, 50 mM Tris–HCl, pH 8.0). SDS-PAGE and Western blot analysis followed standard protocols, using anti-SRF antibody SC-335 from Santa Cruz Biotechnology.

Electrophoretic mobility shift assay

Annealed double-stranded oligonucleotides with *Bgl*II overhangs were labeled with α^{32}P-dCTP using Klenow enzyme (New England Biolabs). Electrophoretic mobility shift assays (EMSAs) with purified protein included 1 × gel shift buffer (20 mM HEPES, pH 7.4, 100 mM KCl, 0.5 mM EDTA, 1 mM DTT, 0.1 mM PMSF, 10% glycerol), 0.5 μg poly dI/dC (Amersham Pharmacia), 2×10^{-7} M SRF, and 5×10^{-10} M labeled probe in a 20 μl total volume and

were incubated at room temperature for 20–30 min. Complexes were resolved on a 4% nondenaturing polyacrylamide gel in 0.6× TBE.

EMSA experiments with nuclear extracts included 50 000 counts (0.4–2 ng) labeled probe and 8 μg total protein in a total volume of 20 μl. Nuclear extracts were prepared as previously described (Osborn et al., 1989). Reactions were incubated in EMSA buffer (20 mM Tris–HCl, pH 7.5, 50 mM KCl with 10% glycerol, 0.1 mM DTT, 0.1 mM PMSF) for 20–30 min at room temperature. Complexes were resolved on a 4% nondenaturing polyacrylamide gel in TGE running buffer (25 mM Tris, 190 mM glycine, 1 mM EDTA, pH 8.3). Unlabeled, double-stranded competitor oligonucleotides were used at 250-fold excess. Antibodies used in supershift EMSA experiments [SC-335 (SRF), SC-866 (E2F4), and SC-59 (SP1), Santa Cruz Biotechnology] were added at a concentration of 2 μg per 20 μl reaction.

DNA binding assays

Quantitation of DNA binding employed similar reaction buffers and conditions, except poly dI/dC was omitted from the reactions, 0.1 μg/ml BSA was added, and protein concentration was varied. Nitrocellulose filter binding assays were performed as previously described (Falcon et al., 1997; Wong and Lohman, 1993). Signal was quantified using ImageQuant software, and data were fit using the following modified Michaelis–Menten equation in SigmaPlot 2000 by nonlinear least squares analysis:

$$R = \frac{Y_m * [\mathrm{SRF}]^n}{K_d^n + [\mathrm{SRF}]^n} \quad (1)$$

Avidin–biotin binding assays

The same oligonucleotide sequences described for EMSA analysis were used in the avidin–biotin assays. Klenow enzyme (New England Biolabs) was used to label double-stranded oligonucleotides with biotin-16-dUTP (Roche) according to manufacturer's instructions. Recombinant SRF (3×10^{-7} M) was incubated with 2×10^{-8} M biotin-labeled vSRE, mvCArG, or cSRE in binding buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 0.5 mM EDTA, 5 mM $MgCl_2$, 0.2% Triton X 100, 5% glycerol, 1 mM DTT, 2.5 ug/ml BSA, and 10 μg/ml sheared salmon sperm DNA) for 2 h at room temperature with tumbling. Streptavidin beads (Sigma) were added, and reactions were incubated at room temperature with tumbling for an additional hour. After four washes in binding buffer, retained complexes were resolved by 8% SDS-PAGE and analyzed by Western blot using the same anti-SRF antibody used in EMSA supershift analysis (Santa Cruz SC-335).

Chromatin immunoprecipitations

Chromatin immunoprecipitations (ChIP) assays were performed according to Upstate Biotechnology's ChIP protocol with a few modifications. Nuclear lysates from 2.5×10^7 Jurkat, HuT102, or MS9 cells were precleared with protein-G beads (Upstate Biotechnology) and rabbit IgG. Anti-SRF antibody (SC-335, Santa Cruz Biotechnology) (5 μg) was incubated with 25 μl washed protein-G beads overnight. Anti-SRF antibody-bound beads were incubated with precleared lysates for 2–3 h at 4 °C with rotation in IP buffer (20 mM Tris–HCl, pH 8.0, 150 mM NaCl, 2 mM EDTA, 1% Triton-X 100, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 1 μg/ml pepstatin, 1 mM PMSF) (Braunstein et al., 1993) and washed sequentially with IP buffer, IP buffer with 0.5 M NaCl, LiCl wash buffer (20 mM Tris–HCl, pH 8.0, 250 mM LiCl, 2 mM EDTA, 0.5% Nonidet-P 40), and TE (Boyd and Farnham, 1999; Boyd et al., 1998; Braunstein et al., 1993). The precipitate was eluted, and crosslinks were reversed. One-tenth of the purified DNA recovered in the eluate was used as template in standard PCR reactions with primers specific for the HTLV-I LTR or c-*fos* promoter. One-tenth of the PCR product was analyzed on a 1% agarose gel and visualized by ethidium bromide staining.

Mutagenesis

An oligonucleotide with the mutant sequence previously described (mvCArG, see above and Fig. 3A) was used as a 5′ PCR primer and a corresponding antisense oligonucleotide was used as a reverse PCR primer in a whole plasmid PCR mutagenesis approach (Costa et al., 1996; Weiner et al., 1994). Deep Vent polymerase from New England Biolabs was used to amplify plasmid pU3RLuc. Cycle parameters were denaturation for 5 min at 95 °C, 10 cycles of 95 °C for 1 min, 55 °C for 2 min, and 72 °C for 3 min, and a final extension at 72 °C for 5 min. Template DNA was then digested with Dpn I (New England Biolabs). Gel purification, phosphorylation, ligation, transformation, and screening followed standard molecular biology protocols (Ausubel et al., 1988; Chenevix-Trench et al., 2002).

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